(19) **Europäisches Patentamt**

European Patent Office

Office européen des brevets

(11) Publication number: **0 219 309**
A1

(12) # EUROPEAN PATENT APPLICATION

(21) Application number: **86307787.1**

(22) Date of filing: **09.10.86**

(51) Int. Cl.⁴: **G 01 N 33/58**
G 01 N 33/569
//G01N33/533

(30) Priority: **11.10.85 US 786917**
01.11.85 US 794945

(43) Date of publication of application:
22.04.87 Bulletin 87/17

(84) Designated Contracting States:
AT BE CH DE ES FR GB GR IT LI LU NL SE

(71) Applicant: **SMITHKLINE BECKMAN CORPORATION**
P.O. Box 7929 1 Franklin Plaza
Philadelphia Pennsylvania 19101(US)

(72) Inventor: **Horan, Paul Karl**
1006 Anna Road
West Chester Pennsylvania 19380(US)

(72) Inventor: **Slezak, Sue Ellen**
209 Valley View Lane
Downingtown Pennsylvania 19335(US)

(74) Representative: **Waters, David Martin, Dr.**
Smith Kline & French Laboratories Ltd. Patent
Department Mundells
Welwyn Garden City Hertfordshire AL7 1EY(GB)

(54) Methods and reagents for performing analyses of subpopulations of particles.

(57) Methods for distinguishing multiple subpopulations of particles in a single sample based upon quantitative differences in the fluoroescence intensity attributable to one or two fluorochromes with which the particles are labelled. The method is used with flow cytometric particle counting techniques to count and sort synthetic particles and biological particles such as the formed elements of blood and other tissue cells. Also disclosed are reagents containing fluorochrome-conjugated antibodies used in the methods.

EP 0 219 309 A1

-1-

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates to a method and composition of matter for using quantitative measurements of fluorescence intensity to measure multiple subpopulations of particles from a single sample of particles by flow cytometric techniques.

DESCRIPTION OF RELATED ART

Flow cytometry is a rapid, high precision technique for analysis and sorting of many different particles, including formed elements of blood and other biologic tissue cells. Using flow cytometry, particles can be counted and sorted by passing a fluid stream containing the particles through a light beam produced by a laser light source. The particles passing through the light beam scatter the illuminating light; measuring the intensity of scattered light at different angles provides information about the size, shape, density, and surface morphology of the particles. Fluorochrome-labelling of the particles to be analyzed provides an often used alternative to relying on differential refraction of light to analyze the particles. When fluorochrome-labelled particles are counted or sorted, the presence or absence of fluorescence within a selected wavelength range emitted by the labelled particles following excitation by the

1   illuminating light is the parameter measured in making the
     analysis. Fluorochrome labelling has advantages
     especially when counting particles of biological origin,
     because, in comparison to methods relying on measuring
5   light refraction, quantitation of specific biochemicals is
     possible.
          For a great many applications, subset analysis,
     defined as distinguishing multiple subpopulations of
     particles in a single sample of particles, would afford
10   great savings in time and expense. Commonly available
     flow cytometers, which include only one laser and two
     fluorescence detection channels, used in conjunction with
     conventional methods, however, are limited to measurement
     of not more than two fluorescent dyes, and thus, can
15   distinguish no more than two subpopulations of particles
     in any one sample. Most efforts to enhance the number of
     subpopulations that can be distinguished in a single
     sample have relied on using highly sophisticated
     instruments. Such instruments contain two or more
20   excitation lasers and a sufficient number of fluorescence
     detection channels to detect fluorescence from three or
     more fluorochromes. Even using these sophisticated
     instruments, the number of subpopulations which can be
     distinguished in a single sample is limited by the finite
25   number of available fluorochromes. Additionally,
     widespread use of these sophisticated instruments,
     particularly for routine clinical diagnosis, is restricted
     by their prohibitively high cost.
          Evidence that the need for a method of subset
30   analysis using widely available instruments remains
     unfulfilled is provided by continuing efforts to develop
     such a method. In United States Patent 4,499,052 to
     Fulwyler, a method of distinguishing multiple
     subpopulations of cells from a single sample of cells is
35

1   described. This method employs several cell-specific
     antibodies having one hundred percent of the antibody
     molecules labelled with different, preselected ratios of
     fluorescein and rhodamine. After reaction with a reagent
5   containing the labelled antibodies, the cells are
     distinguished and counted by comparing the measured
     fluorochrome ratios to the preselected fluorochrome ratios
     and summing the number of cells having each fluorochrome
     ratio.

10   Another method for using widely available
     instruments and fluorochrome-labelled antibodies for
     subset analysis that permits analysis of a limited number
     of subpopulations from a single sample recently has been
     described. Shapiro, H.M., Practical Flow Cytometry,
15   127-128 (1985). According to this method, a sample
     containing several different cell types is mixed with a
     reagent containing three different antibodies having each
     antibody molecule labelled with one fluorochrome.
     Antibodies specific to one cell type are labelled with
20   fluorochrome A, antibodies specific to a second cell type
     are labelled with fluorochrome B, and antibodies specific
     to a third cell type are labelled with the fluorochromes A
     and B such that approximately one-half the third cell
     type-specific antibody molecules are labelled with
25   fluorochrome A and the remaining third cell type-specific
     antibodies are labelled with fluorochrome B. All of the
     third cell type-specific antibodies have the same
     antigenic affinity, and thus the maximal measured
     intensity of each fluorochrome on the third cell type is
30   less than the maximal measured intensity when antibodies
     having the same antigen affinity conjugated to one
     fluorochrome are used alone. After reaction with the
     reagent containing fluorochromes A and B, the subsets,
     upon passing through the excitation laser, emit light of

35

different colors. For example, if fluorochrome A is red
and fluorochrome B is green, the first cell type will emit
only red light, the second only green light, and the third
will emit red and green light. Thus, the three cell types
are counted and separated by segregating red from green
from red and green.

The procedures described in the above references
have in common the use of fluorochrome-labelled antibodies
having one hundred precent of the antibody molecules
labelled with fluorochrome. Since precision dictates that
the cells to be counted be labelled under antibody excess,
cell separation has been restricted to qualitative
distinctions between fluorochrome-labelled cells, that is,
a cell either does or does not emit a certain color or
either does or does not emit a ratio of colors equivalent
to a preselected ratio of colors. Absent from the above
references is a method of distinguishing subsets based
upon quantitative measurements of fluorescence intensity.

SUMMARY OF THE INVENTION

The invention resides in the discovery of a
method for using quantitative measurements of fluorescence
intensity to perform subset analysis. The invented method
makes possible measurement of more than one subset of
particles from a single sample using a single
fluorochrome. Additionally, using the invented method
with two fluorochromes further increases the number of
subsets that are measurable from a single sample.

According to the invented method, each subset to
be measured is labelled with a different amount of a
selected fluorochrome. Then, using flow cytometric
techniques, the number of particles in each subset is
determined by summing the number of particles exhibiting
fluorescence intensities within each measured range

1 between 0% and up to and including 100% intensity (defined
as the maximum fluorescence intensity measurable by the
instrument and instrument settings used). In addition to
determining the number of particles in each subset, the
5 particles may be separated, using standard cell sorting
techniques, based upon measured fluorescence intensity.

In a further aspect of the invention, two
fluorochromes are employed in performing subset analysis.
Each subset to be measured is labelled with one or both
10 fluorochromes so that the amount of each fluorochrome on
the particles of any one subset is between 0% and up to
and including 100% maximal labelling (defined as the
fluorochrome amount that produces 100% fluorescence
intensity). The particle subsets then are counted or
15 sorted based upon quantitative measurements of the
fluorescence intensity of each fluorochrome exhibited by
the particles.

The invention further includes reagents designed
for use in the invented method.

20

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graphic display of the fluorescence
distribution obtained by staining a sample of lymphocytes
with undiluted phycoerythrin-conjugated human suppressor
25 T-cell antibody.

Figure 2 is a graphic display of the fluorescence
distribution obtained by staining a sample of lymphocytes
with undiluted phycoerythrin-conjugated human helper
T-cell antibodies.

30 Figure 3 is a graphic display of the fluorescence
distribution obtained by staining a sample of lymphocytes
with undiluted phycoerythrin-conjugated antibody to human
suppressor T-cells and phycoerythrin-conjugated antibodies
to human helper T-cells diluted with unconjugated
35 antibodies to human helper T-cells.

1   Figure 4 is a two parameter display of the
fluorescence obtained by staining a sample of mononuclear
cells with undiluted phycoerythrin-conjugated antibodies
to human suppressor T-cells, phycoerythrin-conjugated
5   antibodies to human helper T-cells diluted with
unconjugated antibodies to human helper T-cells, and
diluted fluorescein-conjugated antibodies to human T-cells.
          Figure 5 is a two parameter display of the
fluorescence obtained by staining a sample of mononuclear
10   cells with undiluted fluorescein-conjugated antibodies and
undiluted phycoerythrin-conjugated antibodies to human
monocytes, undiluted fluorescein-conjugated antibodies to
human B cells, fluorescein-conjugated antibodies to human
T-cells diluted with unconjugated antibodies to human
15   T-cells, phycoerythrin-conjugated antibodies to human
suppressor T-cells, phycoerythrin-conjugated antibodies to
human helper T-cells diluted with unconjugated antibodies
to human helper T-cells, and phycoerythrin-conjugated
antibodies to human natural-killer cells.
20

DETAILED DESCRIPTION OF THE INVENTION

          The present invention is a method for using
quantitative measurements of fluorescence intensity to
measure multiple subpopulations of particles from a single
25   sample of particles (subset analysis). According to the
invented method, using only one fluorochrome, at least two
fluorochrome-labelled subsets of particles from one sample
may be counted or sorted; using two fluorochromes, from
two to five or more fluorochrome-labelled subsets may be
30   analyzed.

          One technique for using flow cytometry to count
particles requires that the particles first be
fluorochrome-labelled. According to prior art methods,
all of the particles in a sample that are stained with a
35

certain fluorochrome are stained to a similar degree which
is the amount of fluorochrome that renders the
fluorescence intensity of the particles at or near the
maximal fluorescence intensity measurable by the
instrument employed. The sample, including the stained
particles, then is passed through a flow cytometer which
counts stained and unstained particles and generates a
histogram having fluorescence intensity and cell number as
its axes. Figures 1 and 2 are exemplary of the histograms
that are generated when the particles being counted are
cells. As can be seen in Figure 1, for example, a large
number of cells, represented by the peak (A) near the
ordinate, essentially are devoid of fluorescence dye and a
smaller number of cells, represented by the peak (B) very
near the farthest extreme of the fluorescence intensity
scale, are stained intensely with fluorochrome.
Similarly, in Figure 2, the unstained cells are located at
the peak (C) near the ordinate and the stained cells are
at the peak (D) near the far end of the fluorescence
intensity scale. Figures 1 and 2 thus demonstrate prior
art methods of analyzing cells based upon qualitative
differences in fluorescence intensity.

        In contrast to the above methods that rely on
qualitative determinations of fluorescence, the invented
method employs quantitative measurements of fluorescence
intensity to analyze particles. The initial step in the
invented method of counting or sorting multiple subsets of
particles from a single sample of particles is to label
the particles from each subset with an amount of
fluorochrome that differs from the amount applied to the
particles from other subsets. Then, preferably using a
flow cytometer, the fluorescence intensity exhibited by
each particle is measured and the total number of
particles having each of the fluorescence intensity levels

selected by labelling each of the subsets with a different
amount of fluorochrome is determined and the cells are
sorted based upon quantitative differences in measured
fluorescence intensity.

In its least complicated variation, the invented
method is employed to distinguish two subsets using one
fluorochrome. Within the population of particles to be
analyzed, one subset is labelled with a larger amount of
fluorochrome, preferably near the fluorochrome amount that
renders the fluorescence intensity of the subset at or
near the maximum fluorescence intensity measurable by the
instrument and instrument settings being utilized
(saturation-labelled), and the other subset is labelled
with a smaller amount of fluorochrome, preferably, when
analyzing two subsets, the fluorochrome amount that
renders the fluorescence intensity of this subset from
one-half to two-thirds that of the first subset. Once
labelling is complete, the particles are passed through a
flow cytometer for counting and separating based upon
quantitative differences in fluorescence intensity.

Figure 3 is an example of a histogram that is
generated by flow cytometric counting of two subsets of
lymphocytes using the invented method with a single
fluorochrome. The saturation-labelled cells are
represented by the peak (E) near the far end of the
fluorescence intensity axis. The cells stained with a
lesser amount of fluorochrome are represented by the peak
(F) approximately mid-way along the fluorescence intensity
axis. The areas under peaks (E) and (F) provide
measurements of the number of cells within each subset.

To analyze a greater number of subsets according
to the invented method using one fluorochrome, a greater
number of distinguishable fluorochrome label amounts are
chosen and afixed to the subsets to be counted. When

1   three subsets are to be counted, preferably the particles
     are one-third saturation labelled, two-thirds saturation
     labelled, and saturation labelled. To count four subsets
     of particles with one fluorochrome, preferably the subsets
5   are one-fourth saturation labelled, one-half saturation
     labelled, three-fourths saturation labelled, and
     saturation labelled. Similarly, numbers of subsets in
     excess of four are analyzed by progressively increasing
     the number of distinguishable fluorochrome label amounts
10   employed (as defined below).

          According to the invented method, differences in
     fluorescence intensity is the parameter measured to
     perform subset analysis. Thus, subset analysis requires
     that the fluorescence intensities of each of the subsets
15   be sufficiently different to be distinguishable by the
     instrument and instrument settings utilized to make the
     measurements. As can be seen by reference to Figure 3, as
     increasing numbers of different fluorochrome-labelling
     amounts are employed, the distance between a peak
20   representing one subset and the next closest peak
     decreases. Once the fluorescence intensities of the
     subsets becomes so similar that the peaks overlap
     substantially, the efficiency and reliability of the
     subset analysis is compromised. Therefore, using the
25   invented method and one fluorochrome, the number of
     different amounts of fluorochrome label that can be used
     and thus the number of subsets that can be analyzed is
     limited to the number that can be labelled with different
     fluorochrome amounts without causing substantial overlap
30   in the measured fluorescence intensities for each of the
     subsets.

          The number of subsets that can be labelled with
     different fluorochrome amounts without causing substantial
     overlap in measured fluorochrome intensity increases in

35

direct proportion to increases in the dynamic range of the
log amplifier included in the flow cytometer or other
instrument being utilized. Routinely available flow
cytometers are outfitted with amplifiers having a three
log dynamic range; however, amplifiers having a dynamic
range of at least six logs are available and in widespread
use for other applications. When an instrument having a
six-log dynamic range, for example, is used, the maximum
fluorescence intensity detectable by the instrument is
greater than the maximum fluorescence intensity detectable
by a three-log instrument. Thus, the saturation-staining
fluorochrome amount is greater and a larger number of
distinguishable fluorochrome-labelling amounts are
available for labelling subsets to be analyzed.

The number of subsets that can be labelled with
different fluorochrome amounts without causing substantial
overlap in measured fluorochrome intensities, also is a
function of the uniformity with which the particles of the
subsets are fluorochrome-labelled. Thus, a greater number
of subsets of synthetic particles, which can be labelled
more uniformly (low coefficient of variation), are
distinguishable using the invented method than the number
of subsets of biological particles, such as tissue cells,
which are fluorochrome-labelled more heterogenously (high
coefficient of variation). As defined herein,
distinguishable subsets means subsets fluorochrome-
labelled so that the quantitatively measured fluorescence
intensities attributable to the fluorochrome with which
they are labelled or at least one of the fluorochromes if
they are labelled with more than one fluorochrome do not
overlap substantially. Distinguishable fluorochrome
amount means an amount of fluorochrome label afixed to the
particles of a subset of particles that renders the subset
distinguishable from fluorochrome-labelled particles of

1   other subsets based upon quantitative differences in
     fluorescence intensity of the fluorochrome with which the
     particles are labelled or at least one of the
     fluorochromes if the particles are labelled with more than
5   one fluorochrome.

          Using the invented method with two fluorochromes
     further enhances the number of subsets that can be
     analyzed from a single sample. When utilizing one
     fluorochrome, the subsets are separated in one dimension,
10   i.e., fluorescence intensity of one fluorochrome. A
     second fluorochrome makes available another dimension for
     use in separating the subsets. Using two fluorochromes,
     the subsets are labelled with distinguishable amounts of
     one or both fluorochromes and separated based upon
15   quantitative measurements of the fluorescence intensity of
     each of the fluorochromes.

          Figure 4 shows a histogram produced using the
     invented method and two fluorochromes to distinguish two
     subsets of particles wherein the particles are
20   lymphocytes. Each of the subsets, (G) and (H), has been
     labelled with a green-emitting fluorochrome so that the
     green fluorescence intensity is approximately mid-way on
     the fluorescence intensity scale. Subset (G) also has
     been saturation-labelled with a red-emitting fluorochrome
25   and subset (H) also has been labelled with a
     distinguishable amount of the same red-emitting
     fluorochrome. Thus, subsets (G) and (H) are distinguished
     from the essentially unlabelled cells represented by the
     peak (I) near the ordinate and from each other based upon
30   quantitative measurements of fluorescence intensity of
     each of the fluorochromes.

          According to the invented method using two
     fluorochromes, an expansion of the labelling scheme used
     to distinguish two subsets is employed to separate five

35

subsets.   One pattern available for labelling five subsets
with different amounts of two fluorochromes is:

      (i)   a first subset is saturation-labelled with
            one fluorochrome;

     (ii)   a second subset is saturation-labelled with
            a second fluorochrome;

    (iii) a third subset is saturation-labelled with
            the first fluorochrome and saturation-
            labelled with the second fluorochrome;

     (iv)   a fourth subset is saturation-labelled with
            the first fluorochrome and labelled with an
            amount of the second fluorochrome that is
            distinguishable from the amount used in
            saturation-labelling; and

     (v)   a fifth subset labelled with an amount of
            each fluorochrome that is distinguishable
            from the corresponding amount used in
            saturation-labelling with each fluorochrome.

        Figure 5 is a histogram produced by flow
cytometric analysis of five subsets of particles
fluorochrome-labelled with red and green emitting
fluorochromes as described above.   Subset (K) is
saturation-labelled with the green fluorochrome, subset
(N) is saturation-labelled with the red fluorochrome,
subset (J) is saturation-labelled with both fluorochromes,
subset (L) is saturation-labelled with the red
fluorochrome and labelled with an amount of the green
fluorochrome that is distinguishable from the
saturation-labelling amount, and subset (M) is labelled
with an amount of each fluorochrome that is
distinguishable from the corresponding saturation-
labelling amount of each fluorochrome.   As is seen from
Figure 5, the five subsets of particles are distinguished
based upon quantitative measurements of fluorescence

1   intensity of two fluorochromes. The area under each peak
provides a measure of the number of cells in each subset.

Using the invented method with two fluorochromes,
subset analysis on numbers of subsets between two and five

5   and greater than five is performed by labelling each of
the subsets with distinguishable amounts of one or both
fluorochromes and using a flow cytometer to separate and
count or sort the subsets based upon quantitative
measurements of fluorescence intensity. As is found when

10   using the invented method with one fluorochrome, the
maximum number of subsets that can be analyzed using two
fluorochromes is limited to the number of subsets that can
be labelled with different amounts of the fluorochromes
without causing substantial overlap in the measured

15   fluorescence intensities for each subset. With two
fluorochromes, however, the maximum number of subsets
analyzable from a single sample exceeds the maximum number
analyzable using one fluorochrome because subsets labelled
with amounts of one fluorochrome that cause substantial

20   overlap in measured fluorescence intensities are separated
by also labelling these subsets with distinguishable
amounts of a second fluorochrome.

Each of particles within each of the subsets of a
sample of particles to be analyzed according to the

25   present invention must be labelled with a similar amount
of a fluorochrome or fluorochromes which amount is
distinguishable from the amount of fluorochrome or
fluorochromes affixed to the particles of any other
subset. The types of particles which are analyzed include

30   synthetic particles and particles of biologic origin. The
method is useful to analyze microspheres produced, for
example, as stated in U.S. Patent No. 3,790,492, which is
incorporated herein by reference, and to analyze other
polymeric materials. Particles of biologic origin

35

1 analyzed according to the invented method include blood
cells and other formed elements of blood and disrupted
soft tissue cells.

The method of labelling particles with
5 fluorochrome differs depending upon the type of particle
being labelled. Fluorochrome-labelled polymers such as
polyvinyl chloride and polyvinyl pyrrolidine, are produced
by including in the monomer mixture an amount of one or
two fluorochromes sufficient, upon polymerization by
10 standard procedures, to yield polymers having the desired
amount of fluorochromes. Preferably, one of the amounts
of fluorochrome added to the monomer mixture is selected
so that the fluoroescence intensity of the polymer
produced is at or near the upper limit of fluorescence
15 intensity detectable by the instrument and instrument
settings being used. Dilutions of this amount then are
used to label other polymers with a range of amounts of
fluorochrome.

Biological particles, such as formed elements of
20 blood which include red blood cells and red blood cell
precursors, mononuclear cells and mononuclear cell
precursors, and platelets, and other tissue cells, are
fluorochrome labelled by reaction with fluorochrome-
conjugated antibodies, preferably monoclonal antibodies,
25 that have affinity for antigens on the cells of one of the
subsets and do not have significant affinity for antigens
on the cells of the other subsets included in the sample.
Fluorochrome-conjugated monoclonal antibodies having the
required specifity in cell antigen affinity are available
30 from various manufacturers such as Becton Dickinson
Immunocytometry Systems of Mountain View, California,
Coulter Immunology of Hialeah, Florida and others.
Additionally, cell type specific antibodies are prepared
according to standard monoclonal antibody techniques such

35

1   as described in Kohler, G. and C. Milstein, Continuous
Cultures of Fused Cells Secreting Antibody of Predefined
Specificity, Nature 256:495 (1975). Less preferably, the
specific antibodies are prepared by conventional
5   techniques that yield polyclonal antibodies. Once
produced, the specific antibodies are
fluorochrome-conjugated by methods known in the art. See,
e.g., The, T.H. and T.E.W. Feltkamp, Conjugation of
Fluorescein Isothiocyanate to Antibodies: I. Experiments
10   on the Conditions of Conjugation, Immunology 18:865
(1970); The, T.H. and T.E.W. Feltkamp, Conjugation of
Fluorescein Isothiocyanate to Antibodies: II. A
Reproducible Method, Immunology 18:875 (1970); Oi, V.T.,
et al., Fluorescent Phycobiliprotein Conjugates for
15   Analyses of Cells and Molecules, J. Cell Biol. 93:981
(1982).

          As an alternative to direct conjugation of
fluorochromes to the antibody protein, the constant region
of the antibodies are secured to liposomes containing
20   selected amounts of one or two fluorochromes. Liposomes
are prepared and secured to antibodies by published
techniques such as described in Lesserman, L.D.,
Immunologic Targeting of Liposomes in Liposomes, Drugs and
Immunocompetent Cell Functions, ed. C. Nicolau and A.
25   Paraf, Academic Press (1981). Selected amounts of one or
two fluorochromes are loaded into the liposomes by
procedures known in the art. Fluorochrome-conjugating
antibodies using liposomes is preferable when formulating
antibodies having large amounts of fluorochrome such as
30   would be affixed to some of the subsets analyzed using
instruments that include amplifiers having a dynamic range
greater than three logs.

35

1   In another alternative to direct conjugation of fluorochromes to the antibody protein, the constant region of the antibodies are linked to fluorochrome-labelled microspheres. The fluorochrome-labelled microspheres

5   preferably are prepared as described above by incorporating into the monomer mixture a selected amount of one or two fluorochromes. Thus prepared, the fluorochrome-labelled microspheres then are linked to the antibodies by known techniques.

10   The sample of biological particles to be analyzed is fluorochrome-labelled using standard immunofluorescence techniques by adding to the sample one or more fluorochrome-conjugated antibodies that individually have affinity for specific antigens on the cells of the subsets

15   within the sample that is to be separated. The fluorochrome-conjugated antibodies are selected so that each subset is labelled with distinguishable fluorochrome amounts, that is no two subsets are labelled with indistinguishable amounts of both fluorochromes. Any two

20   subsets labelled with indistinguishable amounts of one fluorochrome must be labelled with distinguishable amounts of the second fluorochrome.

       Multiple subsets preferably are analyzed by saturation-labelling one subset with one of the

25   fluorochromes, saturation-labelling a second subset with a second fluorochrome, and saturation labelling a third subset with each of the fluorochromes. Additional subsets are labelled with one or both of the fluorochromes so that they are distinguishable based upon quantitative

30   measurements of the fluorescence intensity of at least one of the fluorochromes.

       Saturation labelling of those subsets in the sample of particles that are labelled with the one fluorochrome optimally is performed by mixing the sample

35

1 with an excess concentration of fluorochrome-conjugated
antibodies having affinity for the antigens specific for
particles of that subset. Saturation labelling of the
particles of those subsets that are labelled with two
5 fluorochromes optimally is performed by mixing the sample
with an excess concentration of first fluorochrome-
conjugated antibodies having specific affinity for
antigens on the cells of the subset and an excess
concentration of second antibodies having specific
10 affinity for antigens on the cells of the subset, which
second antibodies are conjugated to a different
fluorochrome.

Labelling of those subsets that are less than
saturation-labelled with one or both fluorochromes
15 preferably is performed by mixing the sample with a
concentration of fluorochrome-conjugated antibodies less
than that used for saturation labelling and that labels
the cells of the subset with an amount of fluorochrome
that is distinguishable from the amount of fluorochrome
20 affixed to any other subset of particles. To label a
subset of cells with less than saturation-labelling
amounts of two antibodies conjugated to different
fluorochromes, the antibody concentrations must be
selected so that no two subsets of cells are labelled with
25 indistinguishable amounts of both fluorochromes. Because
antibody binding to the cells is more consistent and
predictable when the binding is performed under conditions
of antibody excess, the less than saturation-labelling
concentrations of fluorochrome-conjugated antibodies
30 ideally are prepared by diluting the fluorochrome-
conjugated antibodies with non fluorochrome-conjugated
antibodies having the same antigenic affinity so that the
resulting antibody concentration exceeds that needed to

bind all available antibody binding sites on particles of the subset.

An alternative method for labelling subsets of biologic particles with less than saturation-labelling amounts of one or two fluorochromes is to vary the number of fluorochrome molecules affixed to each molecule of antibody. The maximum number of fluorochrome molecules attached to each antibody molecule is selected so that when biologic particles are reacted with an excess amount of fluorochrome-conjugated antibodies, the particles are labelled with an amount of fluorochrome that renders the fluorescence intensity of the particles at or near the maximum fluorescence intensity measureable by the instrument and instrument settings being used. Particles of the remaining subsets in the sample are labelled with distinguishable fluorochrome amounts by reacting those particles with antibody molecules bearing lesser numbers of fluorochrome molecules. Differences in number of fluorochrome molecules affixed to each antibody molecule are achieved using standard techniques that include varying the fluorochrome concentration in the mixture used to form the fluorochrome-conjugated antibodies and varying the time period that the antibodies being fluorochrome-conjugated are exposed to the fluorochrome-containing mixture.

Various fluorochromes are used in the present invention. Such fluorochromes include fluorescein, rhodamine, Texas red, various cyanine dyes including indocarbocyanines, indodicarbocyanines, oxadicarbocy-anine, thiocarbocyanines, thiodicarbocyanines, merocyanine 540, and safranin O, and sulforhodamine. Additionally, the fluorochromes used in this invention include phycobiliproteins such as phycoerythrin, allophycocyanin, and others listed in U.S. Patent No. 4,520,110 which is

incorporated herein by reference. In a preferred
embodiment of the invention using two fluorochromes, the
fluorochromes are selected so that their excitation
wavelengths fall within the range of wavelengths that are
produced by a single light source, thus enabling the use
of less sophisticated single laser flow cytometers and
other single light source instruments.

The invention includes reagents used to
fluorochrome label the particles analyzed according to the
invented method. The reagent used to perform subset
analysis of biologic cells using one fluorochrome is
comprised of several fluorochrome-conjugated antibodies
each having affinity for antigens specific to the cells of
one of the subsets. Each of the fluorochrome-conjugated
antibodies is present in the reagent in different
concentrations selected so that each subset of cells is
labelled with distinguishable amounts of the
fluorochrome. Sub-maximal fluorochrome labelling of the
cells preferably is achieved by including in the reagent a
sufficient quantity of non fluorochrome-conjugated
antibodies identical in antigen affinity to the
fluorochrome-conjugated antibodies the non-conjugated
antibodies are being used to dilute to form fluorochrome-
conjugated antibody concentrations that result in
labelling each subset with a distinguishable amount of
fluorochrome.

Thus, a reagent used to analyze two subsets with
one fluorochrome using the present invention includes, for
example, a concentration of fluorochrome-conjugated
antibodies having affinity for antigens specific to the
cells of one subset sufficient to saturation label those
cells and fluorochrome-conjugated antibodies having
affinity for antigens specific to the cells of the second
subset diluted with an amount of those same antibodies

unconjugated to fluorochrome sufficient to result in a
concentration of the second subset cell specific
antibodies being approximately one-half to two-thirds the
concentration of the first subset cell specific
antibodies. Reagents used to analyze a greater number of
subsets are prepared by including progressive dilutions of
fluorochrome-conjugated antibodies to each of the
subsets. The concentrations of fluorochrome-conjugated
antibodies included in the reagent, however, must be
sufficiently different to label the cells of each subset
with an amount of fluorochrome label that is
distinguishable from each of the other subsets.

Alternatively, a reagent used to analyze two
subsets with one fluorochrome includes, for example,
antibodies specific to one subset conjugated to a
sufficient number of fluorochrome molecules so that
reacting the subset of particles with the antibodies under
conditions of antibody excess yields saturation-labelled
particles and antibodies specific to the second subset
conjugated to a lesser number of fluorochrome molecules so
that reaction under similar conditions produces particles
having approximately one-half to two-thirds saturation-
labelling fluorochrome amounts. Additional numbers of
subsets are analyzed using reagents having subset specific
antibodies conjugated to progressively fewer numbers of
fluorochrome molecules provided that no two groups of
subset specific antibodies are conjugated to amounts of
fluorochrome that render subsets labelled with such
antibodies indistinguishable.

The preferable reagents used in performing subset
analysis on biological particles with two fluorochromes
preferably include various concentrations of
fluorochrome-conjugated antibodies selected so that using
the reagent to label the cells produces no two subsets of

1   cells that contain indistinguishable amounts of both

fluorochromes. The reagent, therefore, contains

concentrations of fluorochrome antibodies selected so that

upon mixing with the reagent all subsets labelled with

5   indistinguishable amounts of one fluorochrome are labelled

with distinguishable amounts of the remaining

fluorochrome. The various concentrations of fluorochrome-

conjugated antibodies included in the reagent preferably

are prepared by diluting the fluorochrome-conjugated

10   antibodies with non-conjugated antibodies of like

antigenic specificity.

One pattern of fluorochrome-conjugated antibody

concentrations included in a two fluorochrome reagent

designed for subset analysis is:

15   i)   antibodies having affinity for antigens

specific for particles of one subset

conjugated with the first fluorochrome;

         ii)   antibodies having affinity for antigens

specific for particles of a second subset

20   conjugated with the second fluorochrome;

       iii)   antibodies having affinity for antigens

specific for particles of a third subset

conjugated to the first fluorochrome, and

antibodies having affinity for antigens

25   specific for particles of the third subset

conjugated to the second fluorochrome

diluted approximately equally with

unconjugated antibodies of like antigenic

affinity; and

30

iv)   antibodies having affinity for antigens
       specific to particles of a fourth subset
       conjugated to the first fluorochrome
       diluted approximately equally with
       unconjugated antibodies of like antigenic
       affinity, and antibodies having affinity
       for antigens specific to particles of the
       fourth subset conjugated to the second
       fluorochrome diluted approximately equally
       with unconjugated antibodies of like
       antigenic affinity.

This reagent is added to a sample of cells in sufficient
quantity so that each of the differently antigen specific
antibodies is present in sufficient amount to exceed that
needed to label all available antigen-binding sites.
Reagents for analyzing a greater number of subsets are
prepared in a similar manner using progressive dilutions
of the fluorochrome-conjugated antibodies limited by the
requirement that the concentrations of fluorochrome-
conjugated antibodies be sufficiently different so that
when added to a population of cells no two subsets of
cells are labelled with indistinguishable amounts of both
fluorochromes.

       Alternatively, a reagent containing two
fluorochromes includes appropriately selected subset
specific antibodies conjugated to different numbers of
fluorochrome molecules so that upon reaction with the
fluorochrome-conjugated antibodies no two subsets are
labelled with indistinguishable amounts of both
fluorochromes.

       The invention further includes fluorochrome-
labelled particles used as standards to monitor operation
of the instruments used in performing subset analysis and

1   to detect variations in the number of antibody binding
    sites in different samples of biologic tissues. The types
    of particles used include liposomes and synthetic
    polymeric materials such microspheres. The microspheres
5   and liposomes are prepared and fluorochrome-labelled as
    described above. The fluorochrome or fluorochromes used
    to label the particles are selected so that they have
    excitation and emission spectra similar to the
    fluorochrome or fluorochromes used to label the sample for
10 which the particles are being used as standards.
    Preferably, the fluorochrome or fluorochromes used to
    label the particles are stable under refrigeration or in a
    standard preservative solution containing, for example,
    benzyl alcohol or benzalkonium chloride. The particles
15 used as standards preferably are selected such that the
    low angle light intensity, the ninety degree angle light
    intensity, and the size are different from the particles
    contained in the sample to be analyzed following
    standardization.

20   To monitor an instrument used in subset analysis
    or to detect sample-to-sample variations in the number of
    antibody binding sites, a mixture of two or more subsets
    of standard particles labelled with distinguishable
    amounts of one or two fluorochromes is prepared. The
25 number of subsets and fluorescence intensities of the
    subsets of standard particles preferably are selected so
    that they approximate the number of subsets and
    fluorescence intensities of the particles in the sample to
    be analyzed subsequently. The mixture of standard
30 particles then is added to the sample to be analyzed and
    analyzed along with the sample. Alternatively, the
    mixture of standard particles is analyzed in sequence with
    the particles of the sample.

35

1   The following examples are illustrative of the
presently invented method and reagents used with the
method. The examples are presented to describe the
invention rather than to limit its scope as defined above
5   and claimed below.

EXAMPLE 1
Isolation of Nucleated Blood Cells

          In each of the examples below wherein the subsets
10 analyzed are nucleated blood cells, the following
procedure was utilized to separate the nucleated cells
from the remaining constituents of blood.

          Human blood from normal volunteers was collected
by phlebotomy from a peripheral vein using a sodium
15 heparin-containing evacuated container obtained from
Vacutainer Systems of Rutherford, New Jersey. The blood
was obtained from four persons and nucleated cells were
isolated by layering approximately 8 ml. of whole blood on
5 ml. of a sodium metrizoate/Ficoll separation medium
20 (Lymphoprep; Nyegaard and Company, Oslo, Norway). Ficoll
is an inert, non-ionized synthetic, high polymer made by
crosslinking epichlorhydrin and sucrose used as a density
gradient. Tubes containing the whole blood and separation
medium were centrifuged at 400 x gravity for 40 minutes at
25 20° Celcius (C). Then the interface layer was withdrawn
and washed twice in a delbecco's phosphate-buffered salin-
solution, pH 7.2, containing 1% bovine serum albumin and
0.05% sodium azide (PBS-BSA-AZ buffer). The cells were
resuspended in the buffer, counted using a flow cytometer
30 and standard particle counting techniques such as Coulter
counting, and adjusted to a final concentration of 2 x
10^7 cells/ml. Using propidium iodide staining, greater
than 95% of the cells were found viable.

35

EXAMPLE 2
Flow Cytometric Analysis

All analyses using a flow cytometer referred to in the following examples were performed using an EPICS 753 flow cytometer manufactured by Coulter Electronics of Hialeah, Florida. When using the fluorochromes phycoerythrin and/or fluorescein, 500 mw of light at an exciting wavelength of 488 nm was utilized. Also, a 488 nm dichroic mirror and 488 nm band pass for the right angle light scatter signal, a 515 nm interference filter and 515 nm long pass filter to block the excitation wavelength, a 560 nm dichroic mirror to split the fluorescein/phycoerythrin signal, a 590 nm longpass filter for the phycoerythrin signal, a 525 nm bandpass filter for the fluorescein signal, and a 1.5 OD filter for the forward angle light scatter signal were employed. When mononuclear cells were analyzed, gates were set around these cells using right angle light scatter and forward angle light scatter to remove any clumps or debris.

EXAMPLE 3
Fluorochrome-labelling of Biologic Particles

All biologic particles were fluorochrome-labelled by mixing a sample containing the particles with fluorochrome-conjugated antibodies having affinity for antigens specific to the particles of a subset of interest. Fluorochrome-conjugated and unconjugated monoclonal antibodies were purchased from commercial producers.

All labelling of cells was done under standard immunofluorescent staining conditions in 96-well V bottom plates at 4°C. Control wells were set using appropriate unconjugated antibodies or combinations thereof brought to final volume by addition of PBS-BSA-AZ buffer. Fifty

1 microliters of cell suspension containing approximately
1 x 10^6 cells was added to each well with appropriate
amounts of fluorochrome and samples were incubated for 30
minutes.

5   After incubation, first 50 µl of PBS-BSA-AZ
buffer and then 20 µl of fetal calf serum were added to
each well and the plates were centrifuged at 400 x gravity
for 10 minutes at 4°C.   Following supernatant removal, the
cell pellets were resuspended in 200 µl of PBS-BSA-AZ.

10

                        EXAMPLE 4
               Analysis of Two Subsets From a Single
                  Sample Using One Fluorochrome
                  Using phycoerythrin-conjugated monoclonal
15 antibodies, two subsets of mononuclear cells were analyzed
from a sample of mononuclear cells prepared from human
blood.   The subsets analyzed were suppressor T-cells and
helper T-cells.   The subsets were labelled with a reagent
containing phycoerythrin-conjugated anti-Leu-2a monoclonal
20 antibodies which are specific to human suppressor T-cells,
and phycoerythrin-conjugated and unconjugated anti-Leu-3a
monoclonal antibodies which are specific to human helper
T-cells.   The phycoerythrin-conjugated and unconjugated
antibodies were obtained from Becton-Dickinson
25 Immunocytometry Systems, Mountain View, California.

            The phycoerythrin-conjugated anti-Leu-2a
antibodies were obtained in a concentration of 25 µg
purified immunoglobulin/ml. and used without dilution.
Phycoerythrin anti-Leu-3a antibodies obtained in a
30 concentration of 25 µg purified immunoglobulin/ml. were
diluted with unconjugated anti-Leu-3a antibodies in a
concentration of 100 µg purified immunoglobulin/ml. prior
to use.   The anti-Leu-3a antibodies were diluted by adding
1.5 µl of the unconjugated antibody preparation to 13 µl

35

1   of the phycoerythrin-conjugated antibody preparation. The
cell-labelling reagent contained 20 μl of the
phycoerythrin-conjugated anti-Leu-2a preparation, 15μ l of
the diluted phycoerythrin-conjugated anti-Leu-3a
5   preparation, and sufficient PBS-BSA-AZ buffer to bring the
total volumne to 80 μ l.

Figure 3 is a graph of the results obtained by
quantitative measurement of fluorescence intensity of the
labelled mononuclear cells using a flow cytometer equipped
10 with a three-log dynamic amplifier and standard particle
counting techniques. The peak (F) represents the helper
T-cells labelled with the diluted phycoerythrin-conjugated
antibodies and the peak (E) represents the suppressor
T-cells labelled with undiluted phycoerythrin-conjugated
15 antibodies. The area under each of the peaks is a measure
of the number of cells in each of the subsets.

Figure 3 presents the results obtained by
quantitative measurement of fluorescence intensity and
demonstrates that the suppressor T-cells (E) were labelled
20 with an amount of fluorochrome that renders the
fluorescence intensity of these cells very near the upper
limit measurable by the instrument at the settings
utilized. As is indicated by the position of the peak (F)
on the fluorescence intensity axis, the helper T-cells
25 were labelled with an amount of fluorochrome that rendered
the fluorescence intensity of these cells approximately
two-thirds that of the suppressor T-cells.

Preferably, however, the helper T-cells and
suppressor T-cells are fluorochrome-labelled so that the
30 relative fluorescence intensities of these subsets is
reversed. This alternate labelling is achieved by
reacting the sample of mononuclear cells with a sufficient
amount of undiluted phycoerythrin-conjugated anti-Leu-3a
antibodies and appropriately diluted phycoerythrin-

35

1 conjugated anti-Leu-2a antibodies so that the suppressor
T-cells are labelled with an amount of fluorochrome
greater than and distinguishable from the amount with
which the helper T-cells are labelled. Thus labelled, the
5 peaks representing the helper T-cells and suppressor
T-cells appear on the fluorescence intensity scale in
reverse order from that shown in Figure 3.

Thus, using one fluorochrome the helper T-cells
and suppressor T-cells are separated based upon
10 quantitative distinctions of red fluorescence intensity.

EXAMPLE 5
Analysis of Three Subsets From a Single
Sample Using One Fluorochrome
15 Using fluorescein-conjugated monoclonal
antibodies, three subsets of cells are analyzed from a
sample of human blood mononuclear cells. Monocytes,
suppressor T-cells, and helper T-cells are the subsets
analyzed. The monocytes are labelled with
20 monocyte-specific antibodies covalently linked to
liposomes which contain fluorescein immobilized in the
liposome. The amount of fluorescein in the liposomes is
selected so that the fluorescence intensity of the
labelled monocytes is not greater than the maximum
25 intensity measurable by a standard flow cytometer equipped
with a six-log dynamic amplifier and approximately twice
that of the suppressor T-cells. The suppressor T-cells
are labelled with fluorescein-conjugated anti-Leu-2a
antibodies which result in these cells having fluorescence
30 intensities approximately one-half that of the monocytes.
The helper T-cells are labelled with fluorescein-
conjugated anti-Leu-3a antibodies diluted with sufficient
unconjugated anti-Leu-3a antibodies so that the
fluorescence intensities of the helper T-cells are
35 approximately one-half that of the suppressor T-cells.

Then the sample of cells is passed through a standard flow cytometer having a six log dynamic amplifier which segregates and counts the cells of each subset. Labelling the sample of cells as described in this example results in the monocytes having the highest fluorescence intensity, the suppressor T-cells having intermediate fluorescence intensity, and the helper T-cells having the lowest fluorescence intensity with no substantial overlap in the fluorescence intensities of any two subsets.

EXAMPLE 6

Analysis of Two Subsets From A Single Sample Using Two Fluorochromes

Using phycoerythrin-conjugated and fluorescein-conjugated antibodies two subsets of mononuclear cells were analyzed from a sample of mononuclear cells prepared from human blood. The subsets analyzed were suppressor T-cells and helper T-cells. The subsets were labelled with a reagent containing phycoerythrin-conjugated anti-Leu-2a monoclonal antibodies which are specific to human suppressor T-cells, phycoerythrin-conjugated anti-Leu-3a monoclonal antibodies which are specific to human helper T-cells diluted with unconjugated antibodies of like antigenic affinity, and fluorescein-conjugated anti-Leu-4 antibodies which are specific to human T-cells diluted with unconjugated antibodies of like antigenic affinity. As indicated in Example 4, preferably the red fluorescence intensities of the suppressor and helper T-cells is reversed. All of the fluorochrome-conjugated and unconjugated antibodies were obtained from Becton-Dickinson Immunocytometry Systems, Mountain View, California.

1   The phycoerythrin-conjugated anti-Leu 2a
antibodies were obtained in a concentration of 25 µ g
purified immunoglobulin/ml. and used without dilution.
Phycoerythrin-conjugated anti-Leu-3a antibodies obtained
5   in a concentration of 25 µ g purified immunoglobulin/ml.
were diluted with unconjugated anti-Leu-3a antibodies
obtained in a concentration of 100 µ g purified
immunoglobulin/ml by adding 1.5 µl of the unconjugated
antibody preparation to 13 µl of the phycoerythrin-
10   conjugated antibody preparation. Fluorescein-conjugated
anti-Leu-4 antibodies obtained in a concentration of 100µ g
purified immunoglobulin/ml were diluted with unconjugated
anti-Leu-4 antibodies obtained in a concentration of 200µ g
purified immunoglobulin/ml by adding 3 µl of the
15   fluorescein-conjugated antibody preparation to 1 µl of the
unconjugated antibody preparation.

    The cell-labelling reagent contained 20 µl of the
phycoerythrin-conjugated anti-Leu-2a preparation, 15 µl of
the diluted phycoerythrin-conjugated anti-Leu-3a
20   preparation, 5 µl of the diluted fluorescein-conjugated
anti-Leu-4 preparation, and sufficient PBS-BSA-AZ buffer
to bring the total volume to 80 µl.

    Figure 4 displays quantitative measurements of
fluorescence intensity of the subsets of T-cells stained
25   with the reagent of this example. On one axis green
fluorescence is displayed; on the other axis red
fluorescence is displayed. The helper T-cells and
suppressor T-cells have approximately equivalent green
fluorescence intensity, but have red fluorescence
30   intensities sufficiently different so that the red
fluorescence intensities of the suppressor T-cells do not
overlap significantly with the red fluorescence
intensities of the helper T-cells. Thus, based on
quantitative measurements of fluorescence intensity made

35

1   by a standard flow cytometer, the suppressor T-cells and
    helper T-cells, having similar green fluorescence
    intensities, are separated based upon quantitative
    differences in red fluorescence intensities.

5

EXAMPLE 7
Analysis of Five Subsets From a Single
Sample Using Two Fluorochromes

Using seven different monoclonal antibodies, some
10 conjugated to either phycoerythrin or fluorescein and some
    unconjugated, five subsets of human mononuclear cells were
    analyzed from a single sample of mononuclear cells using
    quantitative fluorescence intensity measurements as the
    distinguishing parameter. The subsets analyzed were
15 suppressor T-cells, helper T-cells, natural-killer cells,
    monocytes, and B-cells. Fluorescein-conjugated Bl
    antibodies to human B-cells and fluorescein-conjugated
    Mo2 antibodies to human monocytes were obtained from
    Coulter Immunology. The remaining antibodies were
20 obtained from Becton-Dickinson Immunocytometry Systems.

    The following antibody preparations were employed
    in labelling the five subsets with distinguishable
    fluorochrome amounts. The undiluted preparations were
    used as obtained from the manufacturers after
25 reconstitution according to the manufacturers directions.

    i)   Phycoerythrin-conjugated anti-Leu-11c
            antibodies specific to human natural killer
            cells in a concentration of 50 μg purified
            immunoglobulin/ml;

30 ii)   Fluorescein-conjugated anti-Bl antibodies
            specific to human B lymphocytes in an
            antibody concentration such that 5 μl is
            sufficient to saturation-label 1×10^{6}
            cells in a reaction volume of 100-200 μl;

35

iii) Fluorescein-conjugated anti-Mo2 antibodies specific to human monocytes in an antibody concentration such that 5 μl is sufficient to saturation-label 1×10^6 cells in a reaction volume of 100-200 μl;

iv) Phycoerythrin-conjugated anti-Leu-M3 antibodies specific to human monocytes in a concentration such that 20 μl is sufficient to saturation-label 1×10^6 cells in 100-200 μl reaction volume;

v) Diluted fluorescein-conjugated anti-Leu-4 antibodies specific to human T-lymphocytes prepared by adding 3 μl of conjugated antibodies having a concentration of 100 μg purified immunoglobulin/ml to 1 μl of unconjugated anti-Leu-4 antibodies having a concentration of 200 μg purified immunoglobulin/ml;

vi) Phycoerythrin-conjugated anti-Leu-2a antibodies specific to human suppressor T-cells in a concentration of 25 μg/ml purified immunoglobulin/ml; and

vii) Diluted phycoerythrin-conjugated anti-Leu-3a antibodies specific to human helper T-cells prepared by adding 13.0 μl of conjugated antibodies having a concentration of 25 μg purified immunoglobulin/ml to 1.5 μl of unconjugated anti-Leu-3a antibodies having a concentration of 100 μg purified immunoglobulin/ml

The reagent utilized in differentially labelling the five mononuclear cell subsets included the following amounts of the above antibody preparations:

i)   20 µl of the phycoerythrin-conjugated
        anti-Leu-11c;

ii)   5 µl of the fluorescein-conjugated anti-B1;

iii)   5 µl of the fluorescein-conjugated anti-Mo2;

iv)   20 µl of the phycoerythrin-conjugated
        anti-Leu-M3;

v)   5 µl of the diluted fluorescein-conjugated
        anti-Leu-4;

vi)   20 µl of the phycoerythrin-conjugated
        anti-Leu-2a; and

vii)   15 µl of the diluted phycoerythrin-
        conjugated anti-Leu-3a.

        After labelling a sample of human mononuclear
cells with this reagent, the sample was passed, for
analysis, through a standard single laser flow cytometer
equipped with a three-log dynamic range amplifier.
Figure 5 is the histogram of the five subsets separated
from the sample. From Figure 5 it is seen that the
subsets were segregated in two dimensions by plotting
quantitative measurements of the fluorescence intensity of
the green fluorochrome (fluorescein) on one axis and
quantitative measurements of the fluorescence intensity of
the red fluorochrome (phycoerythrin) on the other axis.
Using these measurements no two subsets overlaped
sufficiently to render them indistinguishable. The cells
of the suppressor T-cell and helper T-cell subsets
represented by peaks (L) and (M), respectively, were
labelled with similar amounts of fluorescein, but
nevertheless were distinguishable because these cells were
labelled with distinguishable amounts of phycoerythrin.
As stated in Example 4, preferably, the suppressor T-cells
and helper T-cells are phycoerythrin-labelled so that the
relative red fluorescence intensities of these two subsets
is reversed from that shown in this example.

1

EXAMPLE 8

Analysis of Seven Subsets From a Single

Sample Using Two Fluorochromes

Using nine different monoclonal antibodies, some

5 conjugated to either phycoerythrin or fluorescein, and

some unconjugated, seven subsets of human nucleated blood

cells are analyzed from a single sample of nucleated blood

cells using quantitative fluorescence intensity

measurements as the distinguishing parameter. The subsets

10 analyzed are suppressor T-cells, helper T-cells,

natural-killer cells, monocytes, B-cells, band cells, and

mature neutrophils.

In labelling the subsets with distinguishable

fluorochrome amounts, fluorescein-conjugated B1 antibodies

15 to human B-cells, and fluorescein-conjugated Mo2

antibodies to human monocytes obtained from Coulter

Immunology are used. The remaining antibodies, except

those to the band cells and neutrophils, are obtained from

Becton-Dickinson Immunocytometry Systems.

20 Antibodies to the band cells and to all neutrophils are

prepared using standard monoclonal antibody techniques.

One of the antibodies has affinity for antigens specific

to all neutrophils, including band cells, (SK&F-MAB-101)

and the second of the antibodies has affinity for antigens

25 specific to the band cells only (SK&F-MAB-102).

Additionally, the antibodies to the neutrophils and band

cells are selected so that they do not compete for binding

to the same antigenic determinant and so that neither of

the antibodies substantially reduces the affinity of the

30 other antibody for its target antigen. SK&F-MAB-101 is

conjugated directly to fluorescein containing liposomes

and SK&F-MAB-102 is conjugated directly to phycoerythrin.

35

1   The following antibody preparations are used as
the reagent in labelling the seven subsets with
distinguishable fluorochrome amounts. Antibodies to the
suppressor T-cells, helper T-cells, T-cells,
5   natural-killer cells, monocytes, and B-cells are used as
described in Example 7. SK&F-MAB-101 antibodies are
conjugated directly to liposomes containing an amount of
fluorescein that renders the green fluorescence intensity
of the neutrophils approximately twice that of the
10   monocytes. SK&F-MAB-102 is conjugated to an amount of
phycoerythrin that renders the red fluorescence intensity
of the band cells approximately equivalent to that of the
monocytes.

          After labelling the sample of human nucleated
15   blood cells with the reagent containing fluorochrome-
conjugated monoclonal antibodies, the sample is passed,
for analysis, through a standard single laser flow
cytometer equipped with a six-log dynamic range
amplifier. The histogram produced by this analysis is
20   similar to that shown in Figure 5, except that the green
fluorescence intensity axis has a wider dynamic range and
the peak representing the neutrophils appears as the most
intensely labelled peak on the green axis, and the peak
representing the band cells has approximately the same
25   green fluorescence intensity as the neutrophils and
approximately the same red fluorescence intensity as the
monocytes.

          In this example, the light scatter gates on the
instrument used are set to include mononuclear and
30   polynuclear leukocytes and to exclude red blood cells and
platelets. As stated in Example 4, preferably, the
suppressor T-cells and helper T-cells are phycoerythrin-
labelled so that the relative red fluorescence intensities
of these two subsets is reversed from that shown in this
35   example.

EXAMPLE 9

Using Fluorochrome-Labelled Particles as Standards

A mixture of microspheres used as standards in association with analysis of five subsets as described in Example 7 contains:

i) microspheres labelled with an amount of 1,1'-didodecylcycloxacarbocyanine (DiO-C(12)-3) sufficient to render the green fluorescence intensity of these microspheres indistinguishable from fluorochrome-labelled B cells from normal donors;

ii) microspheres labelled with an amount of sulforhodamine sufficient to render the red fluoresence intensity of these microspheres indistinguishable from fluorochrome-labelled natural-killer cells from normal donors;

iii) microspheres labelled with an amount of DiO-C(12)-3 and sulforhodamine sufficient to render the green and red fluorescence intensities of these microspheres indistinguishable from fluorochrome-labelled monocytes from normal donors;

iv) microspheres labelled with an amount of DiO-C(12)-3 and sulforhodamine sufficient to render the green and red fluorescence intensities of these microspheres indistinguishable from fluorochrome-labelled suppressor T-cells from normal donors; and

v) microspheres labelled with an amount of DiO-C(12)-3 and sulforhodamine sufficient to render the green and red fluoresence intensities of these microspheres indistinguishable from fluorochrome-labelled helper T-cells from normal donors.

1   After adding the mixture of microspheres to a
sample of human mononuclear cells, the sample is passed,
for analysis, through a standard single laser flow
cytometer equipped with a three-log dynamic range
5   amplifier. The histogram produced upon such analysis is
similar to that shown in Figure 5. Alternatively, the
microspheres are passed through the flow cytometer just
prior to passage of the sample of mononuclear cells and
the operation of the flow cytometer is monitored by
10   comparing the histogram produced upon analysis of the
microspheres to the histogram produced upon analysis of
the sample of cells.

         While the preferred embodiments of the invention
15   are illustrated by the above, it is to be understood that
the invention is not limited to the precise instructions
herein disclosed and extends to all modifications that
fall within the scope of the following claims.

20

25

30

FIGURE 1

ANTI-HUMAN LEU-2A/SUPPRESSOR T-LYMPHOCYTES
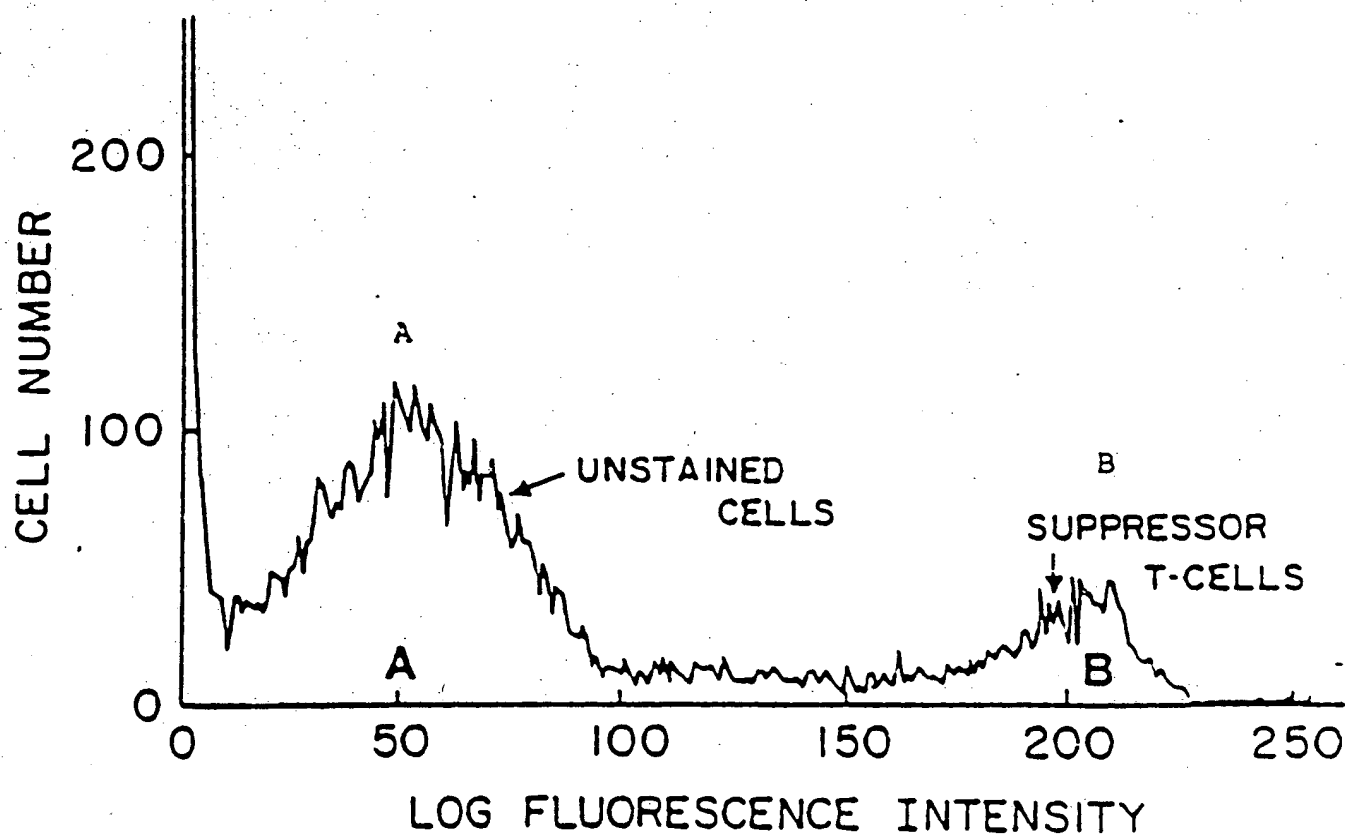
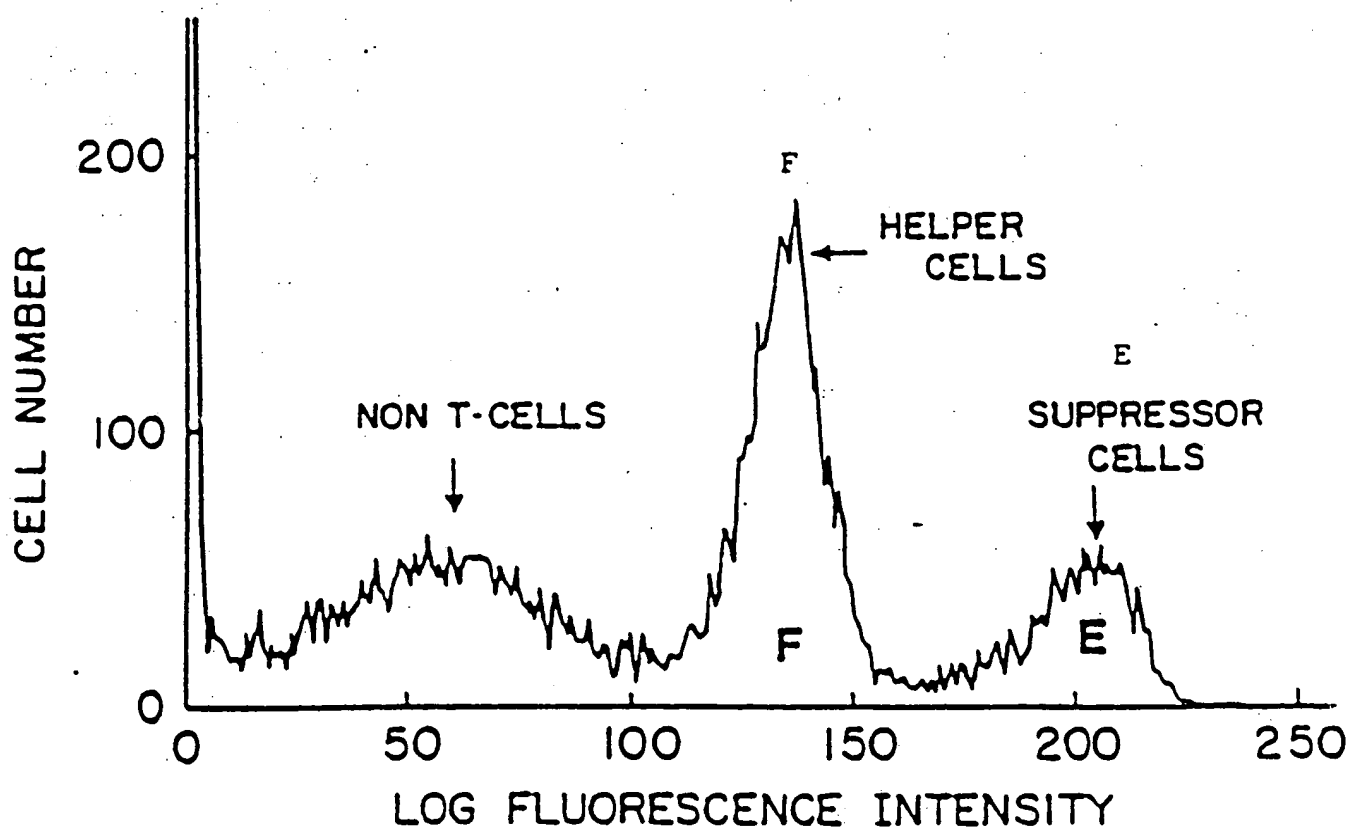FIGURE 2

ANTI-HUMAN LEU-3A/HELPER T-LYMPHOCYTES

(UNDILUTED)
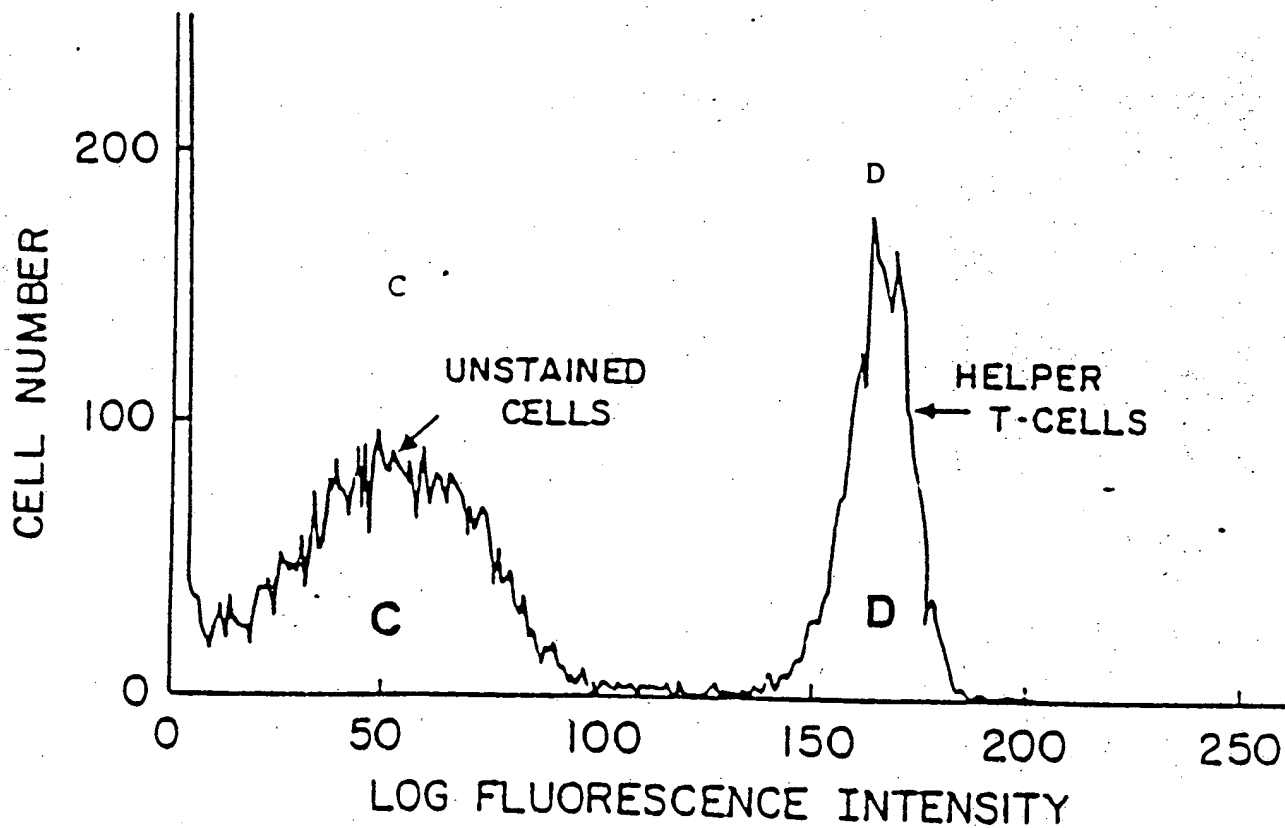
-37(c)-

FIGURE 3

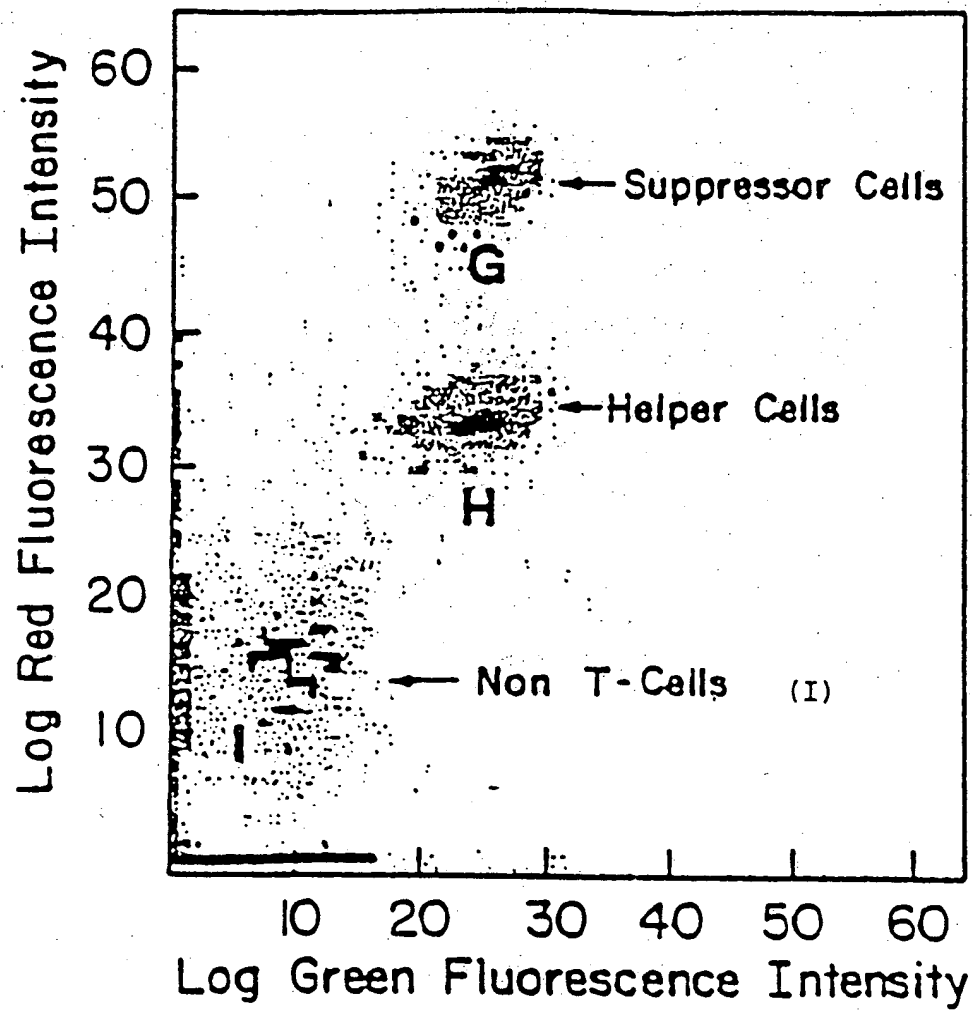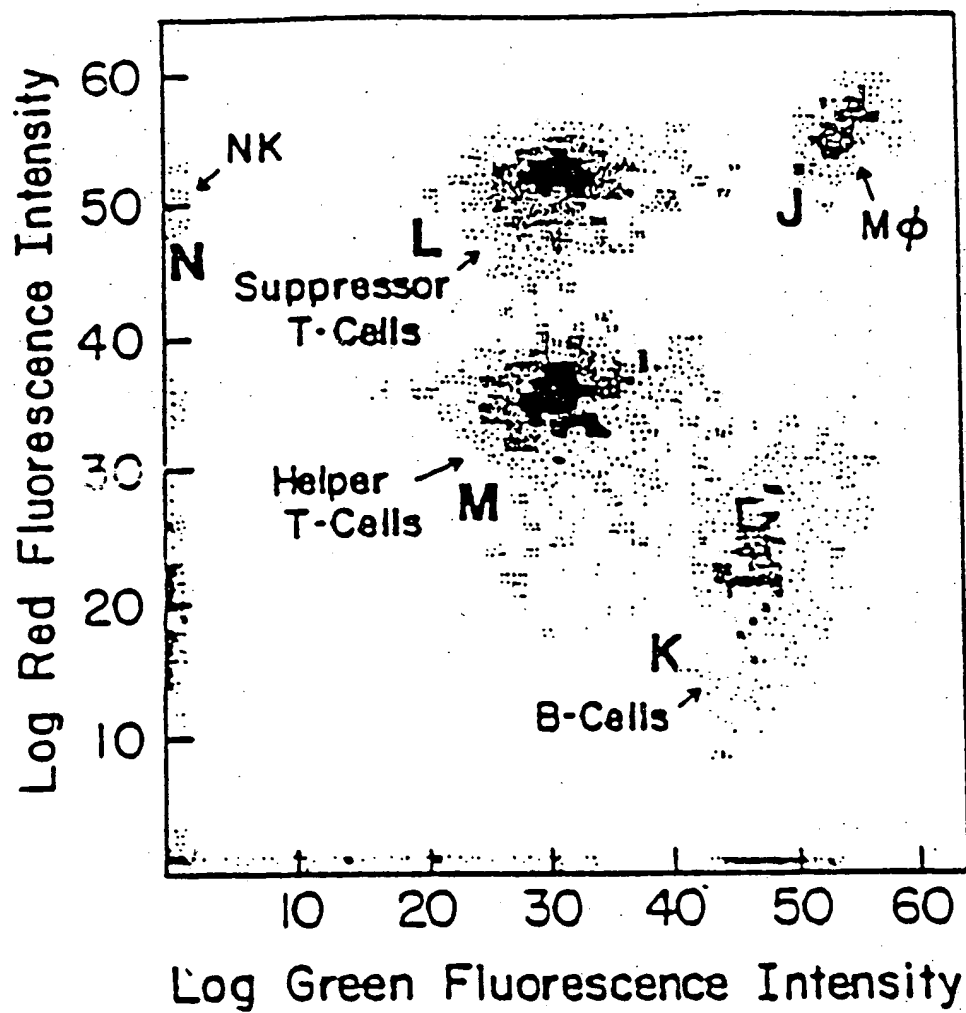ANTI-HUMAN LEU-3A + LEU-2A
HELPER + SUPPRESSOR T-LYMPHOCYTES

FIGURE 4

FIGURE 5

CLAIMS.

1.   A method for distinguishing multiple subpopulations of particles in a single sample of particles that comprises:

labelling the particles of each of the subpopulations with a fluorochrome amount that is distinguishable from the fluorochrome amount with which any other subpopulation of particles is labelled;

quantitatively measuring the fluorescence intensity of the particles of the sample; and

using quantitative differences in the fluorescence intensity of the particles to distinguish the multiple subpopulations of particles.

2.   The method of Claim 1 wherein one fluorochrome is used to label the particles of each of the subpopulations.

3.   The method of Claim 2 wherein the particles in the sample that are fluorochrome-labelled are formed elements of blood.

4.   The method of Claim 3 wherein labelling the particles of each of the subpopulations with a fluorochrome amount that is distinguishable from the fluorochrome amount with which any other subpopulation of particles is labelled comprises affixing fluorochrome-conjugated antibodies to the particles.

5.   The method of Claim 4 wherein the fluorochrome-conjugated antibodies are conjugated to phycoerythrin, fluorescein, rhodamine, sulforhodamine, Texas red, a cyanine dye, allophycocyanine, or other phycobiliproteins.

6.   The method of Claim 1 wherein first and second fluorochromes are used to label the particles of each of the subpopulations with a fluorochrome amount that is distinguishable from the fluorochrome amount with which any other subpopulation of particles is labelled and any two subpopulations labelled with indistinguishable amounts of the first fluorochrome are labelled with distinguishable amounts of the second fluorochrome.

7.   The method of Claim 6 wherein the particles in the sample that are fluorochrome-labelled are formed elements of blood.

8.   The method of Claim 7 wherein labelling the particles of each of the subpopulations with a fluorochrome amount that is distinguishable from the fluorochrome amount with which any other subpopulation of particles is labelled comprises affixing fluorochrome-conjugated antibodies to the particles.

9.   A method for distinguishing five subpopulations of formed elements of blood from a single sample of formed elements using first and second fluorochromes that comprises:

affixing to the formed elements of a first subpopulation a sufficient number of first fluorochrome-conjugated antibodies having affinity for antigens specific for those formed elements to saturation-label the first subpopulation of formed elements with the first fluorochrome;

affixing to the formed elements of a second subpopulation a sufficient number of second fluorochrome-conjugated antibodies having affinity for antigens specific for those formed elements to saturation-label the formed elements of the second subpopulation with the second fluorochrome;

affixing to the formed elements of a third subpopulation a sufficient number of first fluorochrome-conjugated and second fluorochrome-conjugated antibodies having affinity for antigens specific for those formed elements to saturation-label the formed elements of the third subpopulation with each of the fluorochromes;

affixing to the cells of a fourth subpopulation of formed elements a sufficient number of first fluorochrome-conjugated antibodies having affinity for antigens specific for those formed elements to saturation-label the formed elements of the fourth subpopulation with

the first fluorochrome and a sufficient number of second fluorochrome-conjugated antibodies having affinity for antigens specific for the formed elements of the fourth subpopulation to label the formed elements of the fourth subpopulation with a fluorochrome amount that is distinguishable from the saturation-labelling amount; and

affixing to the formed elements of a fifth subpopulation a sufficient number of first fluorochrome-conjugated antibodies and second fluorochrome-conjugated antibodies having affinity for antigens specific for the formed elements of the fifth subpopulation to label those formed elements with an amount of each fluorochrome that is distinguishable from the corresponding amount used in saturation-labelling with each fluorochrome;

quantitatively measuring the fluorescence intensity of the formed elements of the sample by measuring the fluorescence intensity attributable to each fluorochrome; and

using quantitative differences in the fluorescence intensity of one or both fluorochromes to distinguish the five subpopulations of formed elements.

10.   The method of Claim 9 wherein the first and second fluorochromes are selected from fluorescein, phycoerythrin, rhodamine, sulforhodamine, Texas red, cyanine dyes, allophycocyanine, and other phycobiliproteins.

11.   A one-fluorochrome reagent for distinguishing multiple subpopulations of the formed elements of blood from a sample of the formed elements that comprises multiple fluorochrome-conjugated antibodies separately having affinity for antigens specific for one of the subpopulations of formed elements which antibodies are in relative amounts such that after reaction with the reagent the subpopulations of cells are labelled with distinguishable fluorochrome amounts.

1   12.   The reagent of Claim 11 wherein the fluorochrome
is fluorescein. phycoerythrin, rhodamine, Texas red, a
cyanine dye, allophycocyanine, or other phycobiliproteins.
13.   A two-fluorochrome reagent for distinguishing
5   multiple subpopulations of the formed elements of blood from
a single sample of the formed elements that comprises
multiple fluorochrome-conjugated antibodies each separately
having affinity for antigens specific for one of the
subpopulations of formed elements which antibodies are in
10   relative amounts so that after reacting the formed elements
in the sample with the reagent the subpopulations of formed
elements are labelled with distinguishable fluorochrome
amounts.
14.   The reagent of Claim 13 wherein the fluorochromes
15   are selected from fluorescein, phycoerythrin, rhodamine,
Texas red, a cyanine dye, allophycocyanine, and other
phycobiliproteins.
15.   A two-fluorochrome reagent for distinguishing
five subpopulations of the formed elements of blood from a
20   single sample of the formed elements that comprises:
antibodies having affinity for antigens specific
for formed elements of the first subpopulation conjugated
with the first fluorochrome;
antibodies having affinity for antigens specific
25   for formed elements of the second subpopulation conjugated
with the second fluorochrome;
antibodies having affinity for antigens specific
for formed elements of the third subpopulation conjugated
to the first fluorochrome;
30   antibodies having affinity for antigens specific
for formed elements of the third subpopulation conjugated
to the second fluorochrome;
antibodies having affinity for antigens specific
for formed elements of the fourth subpopulation conjugated
35   to the second fluorochrome;

antibodies having affinity for antigens specific
for formed elements of the fourth subpopulation conjugated
to the first fluorochrome diluted with a sufficient number
of unconjugated antibodies of like antigenic affinity to
render the fluorescence intensity of the formed elements
of the fourth subpopulation distinguishable from other
subpopulations labelled with either or both fluorochromes;
and

antibodies having affinity for antigens specific
for formed elements of the fifth subpopulation conjugated
to the first fluorochrome and antibodies having affinity
for antigens specific for formed elements of the fifth
subpopulation conjugated to the second fluorochrome
separately diluted with a sufficient number of unconjugated
antibodies of like antigenic affinity to render the
fluorescence intensity of the formed elements of the fifth
subpopulation distinguishable from other subpopulations
labelled with one or both fluorochromes.

16. A two fluorochrome reagent for distinguishing
five subpopulations of mononuclear cells from a single
sample of cells comprising:

first fluorochrome-conjugated antibodies having
affinity for B cells;

second fluorochrome-conjugated antibodies having
affinity for natural-killer cells;

first fluorochrome-conjugated antibodies and
second fluorochrome-conjugated antibodies having affinity
for monocytes;

first fluorochrome-conjugated antibodies having
affinity for T-cells diluted with a sufficient number of
unconjugated antibodies of like antigenic affinity to
render the first fluorochrome fluorescence intensity of the
T-cells distinguishable from cells of other subpopulations
saturation-labelled with the first fluorochrome;

second fluorochrome-conjugated antibodies having
affinity for helper T-cells; and

1   second fluorochrome-conjugated antibodies having
affinity for suppressor T-cells diluted with a sufficient
number of unconjugated antibodies of like antigenic affinity
to render the second fluorochrome fluorescence intensity of

5   the suppressor T-cells distinguishable from other
subpopulations of cells saturation-labelled with the second
fluorochrome.

       17.   The reagent of Claim 16 wherein the fluorochromes
are selected from fluorescein, phycoerthyrin, rhodamine,

10   sulforhodamine, Texas red, a cyanine dye, allophycocyanine,
and other phycobiliproteins.

       18.   A mixture of fluorochrome-labelled particles
used as standards in the method of Claim 1 that comprises
at least two subsets of particles labelled with

15   distinguishable fluorochrome amounts.

EUROPEAN SEARCH REPORT

0219309

Application number

EP 86 30 7787

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int Cl 4)
A,D	US-A-4 499 052 (M.J. FULLOYLER) * Whole document *	1-18	G 01 N 33/58 G 01 N 33/569// G 01 N 33/533
A	EP-A-0 076 695 (THE BOARD OF TRUSTEES OF LELAND STANFORD JUNIOR UNIVERSITY) * Abstract; page 2, line 6 - page 4, line 12; page 7, lines 11-16; page 9, lines 12 - 36; page 17, line 1 - page 23, line 3 * & US-A-4 520 110 (Cat. D)	1-18	
A	EP-A-0 126 450 (DR. I. TRIPATZIS) * Abstract; page 3, line 19 -page 6, line 5 *	1,4,6, 9,11, 13,15	
			TECHNICAL FIELDS SEARCHED (Int Cl 4)
A	CLINICAL CHEMISTRY, vol. 29, no. 9, September 1983, pages 1582-1586, Washington, D.C., US; M.N. KRONICK et al.: "Immunoassay techniques with fluorescent phycobiliprotein conjugates" * Page 1582, column 1, line 1 - column 2, line 37 *	5,10, 14,17	G 01 N

-/-

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	12-01-1987	HITCHEN C.E.

BEST AVAILABLE COPY

DOCUMENTS CONSIDERED TO BE RELEVANT

Page 2

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
A	CHEMICAL ABSTRACTS, vol. 83, no. 6, 11th August 1975, page 69, abstract no. 44709f, Columbus, Ohio, US; K. DIMROTH et al.; Phosphamethine cyanine dyes with benzimidazolyl substituents", & JUSTUS LIEBIGS ANN. CHEM. 1975, (2), 373-86 * Whole abstract *	5,10, 14,17	
A	EP-A-0 022 670 (ORTHO DIAGNOSTICS INC.)		

TECHNICAL FIELDS
SEARCHED (Int. Cl.4)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	12-01-1987	HITCHEN C.E.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO Form 1503 03 82